

Amendments to the Specification:

Please replace the title at page 1, line 1, with the following new title:

Methods of Treating Disorders Using Modulators of Mitogen-Activated Protein Kinase  
Kinase (MKK) Activity

Please replace the paragraph beginning at page 1, lines 10 to 14 with the following amended paragraph:

This application is a continuation of U.S. application serial no. 09/149,879, filed on September 8, 1998, now U.S. Patent No. 6,174,676; which is a continuation of U.S. Serial No. 09/057,009, filed on April 7, 1998, now U.S. Patent No. 6,541,605; which is a continuation-in-part of U.S. Serial No. 08/530,950, filed on September 19, 1995, now U.S. Patent No. 5,736,381; which is a continuation-in-part of U.S. Serial No. 08/446,083, filed on May 19, 1995, now U.S. Patent No. 5,804,427. The disclosures of the prior applications are incorporated herein by reference in their entirety.

Please replace the paragraph beginning at page 26, line 20 with the following amended paragraph:

GST-JNK1, and GST-ERK2 have been described (Dérjard et al. (1994) supra; Gupta et al. (1995) Science 267:389; Wartmann and Davis (1994) J. Biol. Chem. 269:6695, each herein specifically incorporated by reference). GST-p38 MAP kinase was prepared from the expression vector pGSTag (Dressier et al. (1992) Biotechniques 13:866) and a PCR fragment containing the coding region of the p38 MAP kinase cDNA. GST-MKK3 and MKK4 were prepared with pGEX3X (Pharmacia-LKB Biotechnology) and PCR fragments containing the coding region of the MKK3 and MKK4 cDNAs. The GST fusion proteins were purified by affinity chromatography with the use of GSH-agarose (Smith and Johnson (1988) Gene 67:31). The expression vectors pCMV-Flag-JNK1 and pCMV-MEK1 have been described (Dérjard et al. (1994) supra; Wartmann and Davis (1994) supra). The plasmid pCMV-Flag-p38 MAP kinase was prepared with the

expression vector pCMV5 (Andersson et al. (1989) J. Biol. Chem. 264:8222) and the p38 MAP kinase cDNA. The expression vectors for MKK3 and MKK4 were prepared by subcloning of the cDNAs into the polylinker of pCDNA3 (Invitrogen). The ~~Flag~~ FLAG<sup>®</sup> epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys (SEQ ID NO:16); Immunex, Seattle, WA) was inserted between codons 1 and 2 of the kinases by insertional overlapping PCR (Ho et al. (1989) Gene 77:51).

Please replace the paragraph beginning at page 28, line 29 with the following amended paragraph:

The cells were exposed in the absence and presence of UV-C (40 J/m<sup>2</sup>). The cells were solubilized with lysis buffer (20 mM tris, pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM  $\beta$ -glycerophosphate, 1 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10  $\mu$ g/ml)) and centrifuged at 100,000 x g for 15 minutes at 4°C. MKK3 was isolated by immunoprecipitation. The epitope-tagged protein kinases were incubated for 1 hour at 4°C with the M2 antibody to the ~~Flag~~ FLAG<sup>®</sup> epitope (IBI-Kodak) bound to protein G-Sepharose (Pharmacia-LKB Biotechnology). The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer.

Please replace the paragraph beginning at page 30, line 25 with the following amended paragraph:

In-gel protein kinase assays were performed with cell lysates and JNK1 immunoprecipitates after SDS-PAGE by renaturation of protein kinases, polymerization of the substrate (GST-ATF2, residues 1-505) in the gel, and incubation with [ $\gamma$ -<sup>32</sup>P]ATP (Dérjard et al. (1994) supra). The incorporation of [<sup>32</sup>P]phosphate was visualized by autoradiography and quantitated with a ~~Phosphorimager~~ PHOSPHORIMAGER<sup>™</sup> and ~~ImageQuant~~ IMAGEQUANT<sup>™</sup> soft-ware (Molecular Dynamics Inc., Sunnyvale, CA). The cell lysates demonstrate the presence of 46 kD and 55 kD protein kinases that phosphorylate ATF2 in extracts prepared from UV-irradiated cells. The 46 kD and 55 kD protein kinases were identified as JNK1 and JNK2, respectively.

Please replace the paragraph beginning at page 37, line 18 with the following amended paragraph:

Substrate phosphorylation by p38 MAP kinase was examined by incubation of bacterially-expressed p38 MAP kinase with I $\kappa$ B, cMyc, EGF-R, cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), c-Jun, and mutated ATF2 (Thr<sup>69,71</sup>) and ATP[ $\gamma$ -<sup>32</sup>P] (Raingeaud et al. (1995) J. Biol. Chem 270:7420, herein specifically incorporated by reference). GST-I $\kappa$ B was provided by Dr D. Baltimore (Massachusetts Institute of Technology). GST-cMyc (Alvarez et al. (1991) J. Biol. Chem. 266:15277), GST-EGF-R (residues 647-688) (Koland et al. (1990) Biochem. Biophys. Res. Commun. 166:90), and GST-c-Jun (Dérjard et al. (1994) supra) have been described. The phosphorylation reaction was terminated after 30 minutes by addition of Laemmli sample buffer. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. The rate phosphorylation of the substrate proteins was quantitated by ~~PhosphorImager~~ PHOSPHORIMAGER™ (Molecular Dynamics Inc.) analysis. The relative phosphorylation of ATF2, MBP, EGF-R, and I $\kappa$ B was 1.0, 0.23, 0.04, and 0.001, respectively.

Please replace the paragraph beginning at page 38, line 6 with the following amended paragraph:

Cell extracts expressing epitope-tagged JNK1 and p38 MAP kinase were incubated with a GST fusion protein containing the activation domain of ATF2 (residues 1-109) immobilized on GSH agarose. The supernatant was removed and the agarose was washed extensively. Western blot analysis of the supernatant and agarose-bound fractions was conducted as follows: proteins were fractionated by SDS-PAGE, electrophoretically transferred to an ~~Immobilon-P~~ IMMOBILON-P™ membrane, and probed with monoclonal antibodies to phosphotyrosine (PY20) and the ~~Flag~~ FLAG® epitope (M2). Immunocomplexes were detected using enhanced chemiluminescence (Amersham International PLC). Control experiments were performed using immobilized GST.

Please replace the paragraph beginning at page 41, line 21 with the following amended paragraph:

Immunocytochemistry. Coverslips (22mm x 22mm No. 1; Gold Seal Cover Glass; Becton-Dickinson) were pre-treated by boiling in 0.1 N HCl for 10 minutes, rinsed in distilled water, autoclaved and coated with 0.01% poly-L-lysine (Sigma; St. Louis MO). The coverslips were placed at the bottom of 35 mm multiwell tissue culture plates (Becton Dickinson, UK). Transfected COS-1 cells were plated directly on the coverslips and allowed to adhere overnight in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Gibco-BRL). 24 hours post-transfection, the cells were rinsed once and incubated at 37°C for 30 minutes in 25 mM Hepes, pH 7.4, 137 mM NaCl, 6 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM glucose. The cells were rinsed once with phosphate-buffered saline and the coverslips removed from the tissue culture wells. Cells were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at 22°C. The cells were permeabilized with 0.25% Triton X-100 in phosphate-buffered saline for 5 minutes and washed three times in DWB solution (150 mM NaCl, 15 mM Na citrate, pH 7.0, 2% horse serum, 1% (w/v) bovine serum albumin, 0.05% Triton X-100) for 5 minutes. The primary antibody (M2 anti-FLAG FLAG<sup>®</sup> monoclonal antibody, Eastman-Kodak Co., New Haven, CT) was diluted 1:250 in DWB and applied to the cells in a humidified environment at 22°C for 1 hour. The cells were again washed three times as above and fluorescein isothiocyanate-conjugated goat anti-mouse Ig secondary antibody (Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD) was applied at a 1:250 dilution for 1 hour at 22°C in a humidified environment. The cells were then washed three times in DWB and then mounted onto slides with ~~Gel-Mount~~ GEL-MOUNT™ (Biomedica Corp. Foster City, CA) for immunofluorescence analysis. Control experiments were performed to assess the specificity of the observed immunofluorescence. No fluorescence was detected when the transfected cells were stained in the absence of the primary M2 monoclonal antibody, or mock-transfected cells.

Please replace the paragraph beginning at page 42, line 26 with the following amended paragraph:

Digital images of the fluorescence distribution in single cells were obtained using a Nikon 60x ~~Planapo~~ PLANAPO™ objective (numerical aperture = 1.4) on a Zeiss ZEISS® IM-35 microscope equipped for epifluorescence as previously described (Carrington et al. (1990) in: Non-invasive Techniques in Cell Biology (Fosbett & Grinstein, eds.), Wiley-Liss, NY; pp. 53-72; Fay et al. (1989) J. Microsci. 153:133-149). Images of various focal planes were obtained with a computer controlled focus mechanism and a thermoelectrically cooled charged-coupled device camera (model 220; Photometrics Ltd., Tucson, AZ). The exposure of the sample to the excitation source was determined by a computer-controlled shutter and wavelength selector system (MVI, Avon, MA). The charge-coupled device camera and microscope functions were controlled by a microcomputer, and the data acquired from the camera were transferred to a Silicon Graphics model 4D/GTX workstation (Mountainview, CA) for image processing. Images were corrected for non-uniformities in sensitivity and for the dark current of the charge coupled device detector. The calibration of the microscopy blurring was determined by measuring the instrument's point spread function as a series of optical sections at 0.125  $\mu\text{m}$  intervals of a 0.3  $\mu\text{m}$  diameter fluorescently labeled latex bead (Molecular Probes Inc.). The image restoration algorithm used is based upon the theory of ill-posed problems and obtains quantitative dye density values within the cell that are substantially more accurate than those in an un-processed image (Carrington et al. (1990) supra; Fay et al. (1989) supra). After image processing, individual optical sections of cells were inspected and analyzed using computer graphics software on a Silicon Graphics workstation. p38 MAP kinase was observed at the cell surface, in the cytoplasm, and in the nucleus. After irradiation, an increased localization of cytoplasmic p38 to the perinuclear region was detected.

Please replace the paragraph beginning at page 43, line 26 with the following amended paragraph:

CHO cells were co-transfected with the plasmid pCMV-Flag-Jnk1 and pRSV-Neo (Dérjard et al. (1994) supra). A stable cell line expressing epitope-tagged Jnk1 (Flag FLAG®; Immunex Corp.) was isolated by selection with Geneticin (Gibco-BRL). The cells were incubated with 0, 100, 150, 300, 600, or 1000 mM sorbitol for 1 hour at 37°C. The cells were collected in lysis buffer

(20 mM Tris, pH 7.4, 1% Triton X-100, 2 mM EDTA, 137 mM NaCl, 25 mM  $\beta$ -glycerophosphate, 1 mM orthovanadate, 2 mM pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin) and a soluble extract was obtained by centrifugation at 100,000 g for 30 minutes at 4°C. The epitope-tagged JNK1 was isolated by immunoprecipitation with the monoclonal antibody M2 (Immunex Corp.). The immunoprecipitates were washed extensively with lysis buffer. Immune complex kinase assays were done in 25  $\mu$ l of 25 mM Hepes, pH 7.4, 25 mM  $\text{MgCl}_2$ , 25 mM  $\beta$ -glycerophosphate, 2 mM dithiothreitol, 100  $\mu$ M orthovanadate, and 50  $\mu$ M ATP [ $\gamma$ - $^{32}\text{P}$ ] (10 Ci/mmol) with 2.5  $\mu$ g of bacterially expressed c-Jun (residues 1-79) fused to glutathione-S-transferase (GST) as a substrate. The phosphorylation of c-Jun was examined after SDS-PAGE by autoradiography and PhosphorImager PHOSPHORIMAGER<sup>TM</sup> (Molecular Dynamics Inc.) analysis. JNK1 activation was observed at all concentrations of sorbitol exposure.